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(54) Title: DETECTION OF NUCLEIC ACID POLYMORPHISM

(57) Abstract

This application describes a method of detecting DNA variation by monitoring the formation or dissociation of a complex consisting of: (a) a single strand of a DNA sequence containing the locus of a variation; (b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex; (c) a marker specific for the duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex, which comprises continually measuring an output signal indicative of interaction of the marker with duplex formed from the strand (a) and probe (b) and recording the conditions at which a change in reaction output signal occurs which is attributable to formation or dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a). The method, termed Dynamic Allele Specific Hybridization (DASH), scores nucleotide differences in DNA sequences. Fluorescent markers are convenient as markers to underline variations in fluorescence resulting from denaturization or hybridization of the complex.

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DETECTION OF NUCLEIC ACID POLYMORPHISM

This invention relates to nucleic acid sequence variation and more particularly to the scoring of variants (polymorphisms and mutations) occurring in natural DNA sequences.

In genomes of species such as the human it is estimated that on average 1 in 10^3 nucleotides is variant between any two equivalent chromosomes. Although most such variations will be functionally neutral, a small proportion will underlie human phenotypic differences including the risk of disease. DNA variations may be investigated by determining the extent of hybridisation of allele specific probes against DNA segments containing the locus of the variation. In this way, it is possible to record 'matches' (presence of DNA identical to the probe) and 'mis-matches' (presence of DNA non-identical to the probe) for DNA samples from individuals under investigation. However, a problem with existing methods of this type is that it is difficult to determine an adequately discriminatory hybridisation stringency. An improved method of increased reliability and simplicity is therefore much needed.

The present invention comprises a method of detecting DNA variation which comprises forming a complex consisting of :

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation hybridised to the single strand (a) to form a duplex, and

(c) a marker specific for the duplex structure of (a) plus (b) and which reacts uniquely when interacting within the duplex,

(d) continually measuring an output signal of the extent of the resulting reaction of the marker and the duplex whilst steadily increasing the denaturing environment containing the complex, and recording the conditions at which a change in reaction output signal occurs (herein termed the denaturing point) which is attributable to dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a).

In the assay as defined above the complex is first formed and then denaturing conditions are applied to determine the point at which the duplex dissociates. When heating is employed as the method of denaturing the duplex the reaction is carried out in the following way. The test sample is first cooled to hybridise the oligonucleotide or probe to the target DNA, and then heated steadily in a controlled and monitored fashion to detect the denaturing temperature. It will be appreciated, however, that the formation of duplex DNA is an equilibrium reaction, i.e. a two way reaction. It is therefore possible to reverse the order of the events described above. Thus all the defined components of the reaction can be brought together at raised temperature and then cooled in a similarly controlled and monitored fashion to detect the temperature at which the duplex (and complex) is formed . This may be described as the "annealing" temperature. This can be considered to be equivalent to the "denaturing" temperature, but will actually be a different value due to the complex chemistry involved. This principle of equivalence can of course be applied when denaturing/annealing conditions other than heating are employed for the purposes of the invention.

The method defined in the previous paragraph is one in which the components (a), (b) and (c) are brought together prior to formation of the defined complex and under conditions in which (a) and (b) do not hybridise, whereupon the conditions of their environment are steadily adjusted to cause formation of the duplex and resulting complex, and a reaction output signal is obtained indicative of the occurrence of hybridisation of (a) and (b) (herein termed the annealing point).

More generally, therefore, the invention comprises a method of detecting DNA variation by monitoring the formation or dissociation of a complex consisting of :-

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex,

(c) a marker specific for the duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex,

which comprises continually measuring an output signal indicative of interaction of the marker with duplex formed from the strand (a) and probe (b) and recording the conditions at which a change in reaction output signal occurs which is attributable to formation or dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a).

The invention also comprises a method of detecting DNA variation which comprises bringing together

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex,

(c) a marker specific for the duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex,

the components (a), (b) and (c) being brought together under conditions in which :

EITHER (i) the component (a) hybridises to component (b) and the complex is formed with component (c)

OR (ii) the components (a) and (b) do not hybridise and the complex with component (c) is not formed,

thereafter steadily and progressively adjusting the conditions of the environment, respectively,

EITHER (i) to denature the formed duplex and cause dissociation of the complex,

OR (ii) to cause formation of the duplex and resulting complex,

and continually measuring an output signal indicative of the extent of hybridisation of (a) and (b) and resulting complex formation with (c)

and recording the conditions in which a change of output signal occurs which is indicative of, respectively (i) dissociation of the complex or (ii) formation of the complex.

DNA duplexes can be denatured in a number of ways. The most usual systems employed are raised pH or increased temperature. Thus a controlled steady temperature increase is used to apply denaturing 'pressure' to the duplex, to examine at which point matched and mismatched duplexes denature. As an alternative a controlled steady pH increase can be used. Additionally, the principle of the invention may admit a DNA 'micro-chip' format (sub mm scale assay areas on flat surfaces - with the potential for mounting over electrical chips) in which case it opens the possibility of the use of increased negative electric charge (charge repulsion) to push the DNA strands (also negatively charged) away from the surface. If one partner of the duplex is surface bound, this effect will tend to denature the DNA, as for pH and temperature.

In order to choose alternative signal detection methods, any system that gives a different signal for double stranded and single stranded DNAs can be used as the basis for detecting the denaturing (or hybridising) of the probe plus target DNA duplex. The most well known physico-chemical difference between double stranded and single stranded DNA is the spectrum of UV light absorption caused by these molecular species. Apparatus can be devised to utilise this parameter.

A preferred marker for use in the method defined above is one based on fluorescence. Where a fluorescent marker is used, the present invention comprises a method of detecting DNA variation which comprises forming a complex consisting of :

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide probe specific for one allele of the variation hybridised to the single strand (a) to form a duplex, and

(c) a marker specific for the duplex form of (a) and (b) and which fluoresces when bound to or intercalated within the double stranded DNA,

continually measuring the resulting fluorescence whilst steadily increasing the temperature of the environment containing the complex, and recording the temperature (herein termed the melting temperature) at which a decrease of fluorescence occurs

which is attributable to dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a).

In carrying out the method it will be usual to form a series of two or more complexes of the kind defined, each with a probe specific for a different allele of the variation, and observing their respective transition points so as to distinguish alleles of the variation plus the homozygous or heterozygous state if appropriate. Using fluorescence, the melting temperature is conveniently determined by reference to the negative or positive first or second derivative (differential) of the output signal (fluorescence measurement) curve.

The method of this invention is advantageously carried out with the single strand DNA attached to a support material, most conveniently by a biotin/streptavidin type interaction. The single strand is derived from a double stranded DNA product of PCR amplification of a target sequence. Low complexity sequences, such as cultured viral genomes, purified cloned DNAs etc. can be end-labeled with streptavidin or such and used without specific amplification. It is convenient to work with a PCR product over 100 base pairs or preferably from 40 to 100 base pairs in length. The complex may be formed by adding the probe and marker to the single strand in an appropriate buffer solution.

As indicated above, the assay may be performed with the target DNA bound to a surface. However, the invention is not limited to any one format. Having both target and probe in free solution is also possible. There is also the option of localising both to some shared region. Since the method involves forming a duplex between the two species, if neither or only one is localised, then one has to rely upon diffusion to bring the molecules together. However, by localising the species in mutual close proximity (e.g. both to a surface, or even joining the two together at their ends) the efficiency of the duplex formation can be increased. This is of particular importance if hybridisation is

used as the basis of the assay rather than denaturation. It will also improve the speed of the initial hybridisation for the denaturation based format. We have applied this for directly linked target and probe sequences i.e. one joined to the end of the other with a 'stuffer' sequence of irrelevant DNA between them to constitute a 'hinge'.

The method of the present invention it is not restricted to the use of single oligonucleotide probes. Additional probes can be used which anneal sequentially along the target DNA sequence. These might or might not be placed immediately contiguous i.e. with no gaps. Contiguously located probes will co-stabilise each other by the chemistry of 'base stacking' which is a well established phenomenon. It is also possible to construct a direct physical link between the adjacent probes. All the extra duplex DNA resulting from these extra probes increases the signal level of the assay.

1. Principle of the Method

The method of the invention, termed Dynamic Allele Specific Hybridization (DASH) is a technique that detects and scores single nucleotide differences in DNA sequences. In this assay, one strand of a double stranded DNA (e.g. a PCR product) is bound to a solid surface, and the other strand is removed. An oligonucleotide probe, specific for one version of the variation (an allele), is allowed to hybridize to the bound single strand. Next, an intercalating dye is added which fluoresces specifically in the presence of double stranded DNA (i.e., the oligonucleotide probe hybridized to the DNA sample). The reaction is now heated at a steady rate through a range of temperatures, while continually measuring fluorescence. As the temperature rises, the fluorescence decreases gradually until a temperature is reached where the oligonucleotide probe dissociates from the target DNA. This temperature is known as the melting temperature, or Tm. At this point, there is a rapid decrease in fluorescence.

DNA variations can have two or more alleles. To determine which alleles are present in a given DNA sample, an allele specific probe for each version of the variation may be

assayed against the DNA sample. By comparing the 1st derivative of the fluorescence data from the two probings, it is possible to determine whether one or both alleles are present in the DNA sample.

2. Methodology

In outline form, the method consists of the following:

1. PCR amplification of the test DNA sequence
2. Binding one strand of the PCR product to a surface
3. Elution of unbound DNAs and PCR components
4. Neutralization of pH
5. Hybridization of an Allele Specific Oligonucleotide
6. Removal of excess Allele Specific Oligonucleotide
7. Detection of fluorescence during a heating regime

(7a. Repetition of steps 4-7 for alternative allele probes)

8. Analysis of fluorescence outputs

2.1. PCR amplification

To test genomic DNA, Polymerase Chain Reaction (PCR) is used to amplify a segment of DNA containing a known variation. Ideal conditions involve amplifying a short PCR fragment (from 40-100bp), with 18-30 nucleotide long primers. One primer is biotinylated at its 5' end, allowing binding to a solid surface in a later step. Taq Gold, or other "Hot Start" type PCR conditions are used to limit primer dimer artefacts as much as possible. Effective PCR buffer conditions are as follows, with cycle times and numbers appropriate for the particular DNA fragment in question:

Primer 1 (non-biotinylated)	100ng
Primer 2 (biotinylated)	50ng
DMSO	5%
Nucleotides	5.0 nmoles each dNTP

PCR Buffer	to 1x.
Enzyme (e.g. Taq Gold)	0.75 units
Water to a total volume:	25 µl

To limit the amount of non-incorporated biotinylated primer (which competes for binding sites on the solid surface), it was determined that 20ng of biotinylated primer is sufficient. With a ratio of 20ng biotinylated primer to 100ng of non-biotinylated primer, the PCR product formation is still efficient, and the lower concentration of biotinylated primer decreases competition for streptavidin sites when binding to the solid surface.

PCR products of longer lengths (over 100 base pairs) work also, but there are some considerations. With longer PCR products, the variation should be located towards the biotinylated primer as there will be less kinetic motion at this end. Secondary structures can inhibit efficient binding of the probe and should be avoided. Also, the binding efficiency of long PCR products to the plates is reduced, presumably associated with secondary structure complications, as well as slower kinetics of molecule diffusion.

Short PCR products (40-100bp) are preferable for several reasons. Less primer dimer artefact is seen with short PCR products. In addition, the overall efficiency of PCR is often superior when amplifying short products. If the binding capacity of the solid surface can be increased sufficiently, multiplex PCRs can be considered for use in the DASH assay. The short PCRs assists both in the efficiency of the multiplex PCR, and in the binding to the surface.

2.2. Binding one strand of the PCR product to a Solid Surface

The current binding surface format used is a 96 well microtitre plate that has been coated with steptavidin (available from various manufacturers). The total volume (25µl) from one PCR is placed at room temperature in a well of the streptavidin coated plate, along with 25µl buffer I (see sections 2.2.1-2.2.3 for buffer descriptions). The PCR

product then becomes physically attached to the plate via the biotin label on the PCR product binding to the Streptavidin coating on the plate. Binding is left to proceed for anywhere between 5 minutes to 24 hours. Since the binding is 90% complete within one hour, the maximum efficiency is usually achieved after 30 minutes to 2 hours. Typically, less than 20% of the PCR product becomes bound to the plate, even at maximum efficiency. The binding solution can thus be removed and placed in a second well, which will also be completely saturated.

It is important in this step, as with all the following steps, that there are no air bubbles in the reaction tubes (wells on the microtitre plate). Air bubbles interfere with reactions between the solution and the surface of the microtitre plate, and should be removed before each incubation step. This can be done with a pipette tip, or tapping the well with a finger. In addition, it is necessary to remove as much as possible of the volume of solutions from the reaction tubes between steps. The reaction tube should appear "empty" before proceeding to the next step, with no visible solution left in the bottom of the well.

2.2.1. Hepes buffer (buffer I)

Buffer I consists of 100mM Hepes, 50mM NaCl, 10mM EDTA, pH 7.8. There is an important reason why this buffer was chosen. It tends to dramatically standardize/normalize Tms based on the oligonucleotide length, regardless of G+C content. For example see the data given for Figure 7.1.4. Many other buffers allow sufficient allele discrimination, however the absolute Tm's observed will vary greatly depending upon the G+C content.

2.2.2. Alternative Buffers & pH

SSPE, SSC, TEN, TES, MES, and Phosphate buffers were tested, and all maintained the integrity of the experiment. The different buffers vary the observed Tms of oligonucletide/target DNA duplexes. The relationship between the Tm's of the matched

oligo probe to a DNA target compared to the mismatched oligo hybridized to a target remained fairly constant at ~8-10°C. The above buffers supported a pH range from 6.0 to 9.8. There were no significant pH affects detected in this range. Below pH 6.0, the DNA analyzed tended to degrade, and at pH 10.0 and above the Tm was decreased to close to room temperature, preventing effective fluorescence measurement.

2.2.3. Buffer/Salt Concentrations

For the above buffers, a range of buffer and NaCl concentrations (1mM to 400mM) have been tested. At low salt concentrations, the observed Tm decreased as the buffer concentration increased. For example, in Hepes buffer at concentrations 1, 10, 40, and 100mM with 0.0M NaCl, The Tm values decreased from 88°C, 84°C, 78°C, and 72°C respectively in one experiment.

At NaCl concentrations above 10mM, the salt concentration rather than the buffer concentration becomes the major factor affecting the Tm values. Like buffer concentration, increasing NaCl concentration has the tendency to decrease the Tm. A range of NaCl concentrations were tested from 1mM to 800mM. At concentrations above 200mM, the data becomes difficult to interpret (the curves were marked by random fluctuations).

2.2.4. Plastic-ware

The microtitre plates and tubes employed must be made from fluorescence free plastic, and thus provide no additional background to the assay. The microtitre plates and tubes are also frosted to eliminate any fluorescence that may be detected from the outside of the tube. Thus, the plate and caps offer virtually no background to the experiment. Almost all background fluorescence is accounted for by the physical apparatus (excitation/detection equipment).

2.3. Elution of second strand of the PCR product

Once the PCR product has bound to the plate, the non-bound PCR reagents are aspirated away, and 50µl of NaOH solution is added. This denatures the PCR product, leaving single stranded DNA attached to the plate via the biotin/streptavidin interaction. 0.05M NaOH was determined to be the minimum concentration of NaOH needed to reliably denature the double stranded PCR product, however 0.1M allows room for concentration inaccuracies, and does not interfere with subsequent reactions. We allow 0.5-5.0 minutes for full denaturation of the PCR product in 0.1M NaOH. Longer times are not necessary, but have no deleterious effects. The elution solution is aspirated away to remove all residual PCR components (non-incorporated primers, nucleotides, the enzyme, etc.), as well as the non-biotinylated PCR product strand.

2.4. Neutralization of pH

A solution of buffer I is prepared including a fluorescent dye specific for double stranded DNA. We currently use 'SYBR Green I' dye. The optimal concentration of this dye in the DASH assay is 1:10,000. Slight variations in dye concentrations do not affect the results. However, there are some characteristics of the SYBR Green I dye that should be noted (see sections 2.7.2., 3.1. and 3.2.). 50µl of the Buffer I/dye solution is then added to the reaction tube. This buffer will both neutralize any remaining NaOH, and serve as the hybridization buffer in the next step. There should be minimal delay time before proceeding to the next step to minimise the opportunity for formation of secondary structures in the test DNA molecules.

2.5. Hybridization of an Allele Specific Oligonucleotide Probe

The SYBR Green I dye is included in the hybridization solution of the last step as it stabilizes the interaction between the oligonucleotide probe and the test DNA. 30pmols of oligonucleotide probe (in 1µl volume) is added to the reaction tube. The probe can alternatively be added as part of the neutralization buffer. This amount of probe allows hybridization to be completed within seconds, therefore there is no practical minimum

incubation time for this step. Optimal results are obtained by performing a heating plus cooling step (heating to greater than 50 ^{o}C and cooling steadily over ~15 minutes to room temperature), rinsing the wells clear of unbound probe, and re-filling with 50µl of the Buffer I/dye solution. Lower amounts of probe necessitate longer incubation periods, while higher concentrations do not decrease the necessary time for annealing. For further probe details, see section 2.5.1.

2.5.1. Probe Design and Use

Probe lengths of 13-25 base pairs have been tested, on three different variant loci. Allele discrimination is possible at all these lengths, however the optimal probe length was determined to be 15 basepairs. The 13mer probes denatured at temperatures close to room temperature, and were determined to be non-ideal for this technique. The 25mer achieved high fluorescence intensity (as fluorescence is a function of double strand DNA length), but allele discrimination was minimized. The 15mer probe allowed sufficient fluorescence intensity and high discrimination between alleles.

The position of the variation in the hybridization probe was also examined. With a 15mer probe, it was determined that the variant position gives the best discrimination when located in the central third of the probe. For single base variations the variant position is best placed at the central position. If the variant position is moved two bases from the centre, the assay is less discriminatory.

In order to rapidly hybridize the probe to its DNA target on the solid support, 30pmols was determined to be effective. This necessitates removal of the probe before fluorescence detection. An alternative was tested involving much lower amounts of probe (1-5pmol) for hybridization, and subsequent processing without removal of the excess probe. Although allele distinction was achieved, the fluorescence values were low and the results were highly variable. With a higher binding capacity on the plate, this strategy may prove effective, decreasing the number of steps involved in the assay.

2.6. Removal of Excess Probe

The hybridization solution is next aspirated away to remove unhybridized probe molecules, and 50µl of the buffer I/dye is added. The experiment is then ready for heating and fluorescence detection.

2.7. Fluorescence Detection and Heating Regime

The microtitre plate is placed in a heater/detector apparatus. Several devices are available which allow coincident temperature modulation and fluorescence detection as required to produce melting temperature profiles. These include a purpose-built "DASH machine" from Hybaid which allows automated scoring of alleles, and the Perkin-Elmer 7700 (Taqman) machine which was used for generation of the data presented in this document. The sample plate is heated from ~25°C to ~90°C, while continually monitoring fluorescence. Most samples denature around 65 ^{o}C $\pm$10 ^{o}C. Heating rates may vary at least between 0.01 to 1.0 ^{o}C per second with little loss of allele discrimination. We typically run assays at a rate of 0.1 ^{o}C per second. For details regarding the hardware of the Taqman device see section 2.7.1.

2.7.1. Perkin Elmer 7700 (Taqman) Sequence Detector

The detection device must detect the emission spectra given off by the double strand DNA specific fluorescent dye and keep track of the temperature at which the fluorescence data points were extracted. The excitation light source frequency must correspond to the requirements of the dye used in the DASH assay. For example, for dyes such as SYBR Green I excited near the 488nm frequency, an Argon laser or a halogen lamp (filtered for the 488nm frequency) is sufficient to excite the dye molecules. The Taqman is equipped with an argon laser that excites the fluorescent molecules. A filter is in place, removing all other wavelengths in the argon laser spectra, except for the 488nm wave length.

The CCD camera on the Taqman detects a frequency range between 500 and 660nm. The fluorescent signals are recorded into 5nm "bins". Thus "bin1" would contain the fluorescence data from 500-505nm. With the current arrangement, the light frequency range we use for the DASH assay is bin9 (545-550nm), though this is not the only bin that is effective.

2.7.2. Fluorescent Dye

The dye employed must have a specificity such that its fluorescence in the presence of double stranded DNA is at least 10 times greater than when in free solution. We currently use 'SYBR Green I' dye which has a specific signal increase of ~1000 fold.

The optimal concentration of SYBR Green I dye depends directly on the amount of DNA present in the sample. We refer to this as the 'SYBR Green effect'. If the amount of dye used is below or above the optimal concentration for the given amount of DNA, the overall fluorescence observed in the assay will be reduced. For this assay, the optimal dye dilution is 1:10,000.

Alternative dyes have also been tested, and Vistra Green (Amersham) appears to have near identical properties to SYBR Green I, and could be used as an alternative dye for the DASH assay. Other dyes, such as acridinium orange and ethidium bromide gave high background fluorescence and are therefore not suitable for DASH. Other dyes, such as Yo-Pro I and To-Pro I have not been assayed due to the inappropriate light source plus filter combination in the Taqman device.

2.7.3. Assay Solution Additives

An array of different additives were screened for effects on the assay. Common destabilizing agents, like formamide, were screened and shown to be non-beneficial to the assay. In addition, hybridization reagents like Tetra-methyl Ammonium Chloride (TMAC), Bovine Serum Albumin (BSA), and Dextran Sulphate were tested, and again

found to cause irregularities in the assay. Ionic detergents, even in trace amounts, destroyed the fluorescent signal completely. Non-ionic detergent showed no negative effects, except for the tendency to produce bubbles in the reaction tubes. With non-ionic detergents, much more care was needed to make sure bubbles did not remain in the reaction tubes at the various steps in the DASH assay. Dimethyl Sulfoxide was the only additive that was found to be beneficial, in that it can be added at a 50% level or less to decrease the observed Tm values.

2.8. Analysis of fluorescence outputs

Interpretation of the output fluorescence versus temperature graphs is conveniently achieved as follows. A graph of the primary data is used to determine general information regarding how well the assay performed, i.e. the level of fluorescence and which samples may have failed. To score alleles, the results of a series of samples (different DNAs hybridized with the same allele specific probe) are plotted together according to the negative of the first derivative of the fluorescence values. For convenience, DNAs of known genotypes can be included in the series. For two alleles, two distinct peaks should be observed on the graph. These peaks correspond to maximal rates of fluorescence decrease (denaturing probe/target duplexes) in the primary data. The two peaks thus correspond to the Tms of the probe/target "matched" and "mismatched" duplexes.

The Tm peaks will be separated by at least 8°C. The higher temperature Tm peak indicates the presence in a given DNA sample of the sequence corresponding to the allele specific probe used in the experiment. This can be termed a 'match', as the allele specific probe matches perfectly to molecules in the test DNA. The lower temperature Tm peak indicates a 'mismatch', i.e. the presence of hybridising sequences in the test DNA that are similar but non-perfectly matched to the allele specific probe used in the experiment. For the typical case of a two allele system, this 'mismatch' will be the allele

not represented by the probes' sequence. Often a single sample will give both peaks, indicating that it is heterozygous for the two tested alleles.

The bound DNA samples may be reprocessed through steps 4-7 of the procedure using a probe comprising the second (or several subsequent) allele sequence(s), and the data is analyzed as above. By comparing the two sets of data, it is possible to determine with high reliability which alleles are present in the DNA samples. If a DNA sample scores a 'match' with the probe specific to allele 1, and a 'mismatch' with the probe specific to allele 2, the DNA sample is scored 'homozygous allele 1'. If the DNA sample scores a 'mismatch' with allele 1, and a 'match' with the allele 2 probe, then the sample is scored 'homozygous allele 2'. If the DNA sample is scored a 'match' for both alleles, then the sample is scored 'heterozygous for alleles 1 and 2'. For examples of primary data, 1st derivative, and 2nd derivative graphs, see section 7.1.

3. Novel Discoveries

3.1. Melting Temperature (Tm)

In this assay, the temperature at which the oligonucleotide probe disassociates from the DNA target is determined by interactions between the dye, the buffer, and the salt concentrations. SYBR Green I dye stabilizes the probe/DNA duplex structure, raising the Tm with increases in Dye concentration. Increasing Salt and Buffer concentrations decrease the Tm, presumably by decreasing the potential of the dye to bind(and so stabilise) the duplex DNA structure.

3.2. Dye effects

SYBR Green I intercalates into double stranded DNA structures and thereby increases the Tm distinction between matched and mismatched duplexes. The Tm difference between a completely 15mer oligonucleotide probe hybridized to it's perfectly matched DNA target compared to a target mismatched at the central position is roughly 8°C. The

expected difference based on melting temperature calculations for normal DNA solutions would be around 2-3°C (depending on the DNA nucleotide sequence of the probe). The current DASH format thus optimises the potential for allele discrimination.

SYBR Green I also produces an effect wherin fluorescence is dependant on the double stranded DNA concentration and the concentration of the dye. Thus, for a constant DNA concentration, titrating SYBR Green Dye levels increases the fluorescence signal until a point is reached whereafter the fluorescence signal will decrease. We call this the 'SYBR Green Effect'. The concentration of dye used in this assay (1:10,000 dilution) is optimal for the amount of DNA that can be bound to the typical microtiter plate wells at this time.

3.3. Hepes Buffer

Hepes, as the base in the hybridization buffer, has some unique characteristics in this assay. In this buffer and dye combination, the Tm of the probe/target DNA complex is a function almost solely of the length of the probe. Variations in the DNA sequence context, or the G+C content, will not alter the observed Tm. This is of extreme importance for robustness of the DASH assay.

3.4. Dimethyl Sulfoxide (DMSO)

DMSO can be added to the hybridization buffer to lower the Tm of the oligonucleotide probe/target DNA complex without compromising the integrity of the assay. The sole affect is to lower the Tm of both allele Tm's without affecting the Tm difference between them.

3.5. Ionic Detergents

Use of ionic detergents, such as Sodium Dodecyl Sulphate, as low as 0.1% concentration, will completely destroy the fluorescent signal, presumably by interacting with the dye.

4. DASH Assay Components

4.1. Dyes

SYBR Green I Dye from Molecular Probes.

4.2. Plates

Microtitre plates from various suppliers. Micro Amp Optical plates, caps, and tubes from Perkin Elmer are designed especially for fluorometric measurements.

4.3. Oligonucleotides

PCR primers and allele specific oligonucleotide probes from Interactiva Biotechnologie, all HPLC purified in order to ensure maximal quality.

4.4. Fluorescence monitoring device

The Hybaid DASH system (Hybaid Limited, UK); ABI 7700 (Perkin Elmer; used in assays presented) or other detection temperature controlled device.

4.5. Buffers

All the components of buffers were from Sigma.

4.6. Software

The software developed to analyze the raw data, first derivative, and second derivatives of sample data was written by Kin-Chun Wong (Uppsala).

5. An Alternative Assay Format

An alternate format of binding the oligonucleotide probe to the plate followed by hybridization of the PCR product should be possible, but several technical problems arose with this design when tried. First is the problem of hybridising the double stranded PCR product to the bound probe. Simple heat denaturation of the PCR product followed by cooling in the probe coated assay wells is insufficient. Presumably this is because of

displacement reactions, i.e., the PCR product reforms it's double strand and displaces any probe hybridized to the target sequence.

To eliminate the complications caused by displacement reactions, we attempted to generate single stranded DNA from the PCR product for hybridization. For this we used unequal amounts of the two primers in the PCR, thus theoretically producing an excess of one strand of the PCR product (asymmetric PCR). This did improve the assay, but only to a small degree. Importantly, optimal PCR conditions varied compromising the robustness of the DASH assay.

The long length of the PCR products in free solution also presents a problem. This could slow reaction kinetics, and potential secondary structures would interfere with the hybridisation reaction. In addition, once the long molecule is hybridized to the short fixed probe, there appears to be premature disassociation of the DNA/probe complex as thermal energy is added. In this format, it was found to be impossible to distinguish between alleles because all fluorescence signal is lost at very low temperatures. This is probably due to the hybridized PCR fragment having long non-hybridized tails sticking out into the solution. As the temperature rises, the long molecules will be pulled off by the solution kinetics rather than denatured according to Tm properties of the hybridized duplex.

Optimisation of the probe bound format will require further experimentation, involving PCR conditions, buffer components, annealing strategies, as well as other parameters. The problems concerning production of single stranded DNA molecules for hybridization, and the kinetic considerations are avoided by using the DNA molecule bound format. The non-biotinylated strand is simply eluted away for single strand DNA production, and the kinetic limitations involving premature displacement are not observed.

6. Worked Example of DASH Analysis : Detection of allelic versions of a bi-allelic single nucleotide polymorphism in the human NDUFB4 gene

A DASH experiment was performed on a single nucleotide polymorphism in the human NDUFB4 gene which is located on an autosome. This is illustrated in figure 1 as 'DNA sequence 1' and it comprises a bi-allelism between 'A' and 'G' nucleotides. Three human genomic DNA samples, X, Y and Z, were employed that were known from earlier sequence analysis to be homozygous for the 'A' allele, homozygous for the 'G' allele, and heterozygous for these alleles, respectively.

6.1. Polymerase chain reaction (PCR)

PCR was performed on 50ng aliquots of DNA samples X, Y and Z, using the PCR primers presented figure 1 as 'DNA Sequence 2' and 'DNA Sequence 3'. Reaction conditions were as follows; 25ml total volume comprising 20ng 'DNA Sequence 2' primer, 100ng 'DNA Sequence 3' primer, 0.75u AmpliTaq-Gold polymerase (Perkin-Elmer), 10% dimethylsulphoxide, 1x Perkin-Elmer PCR-buffer (including 1.5mM $MgCl_2$) and 0.2mM each of dGTP, dATP, dTTP and dCTP. Thermal cycling employed a TouchDown™ Temperature Cycling Device(Hybaid Ltd) and the following cycle conditions: 1x (10 minutes at 94°C, 30 seconds at 50°C, 30 seconds at 72°C), 17x (15 seconds at 94°C, 30 seconds at 50°C, 30 seconds at 72°C), 18x (15 seconds at 94°C, 30 seconds at 50°C, 1 minute at 72°C). This produced 48 base pair long PCR products that spanned the polymorphic locus and possessed a biotin moiety on the 5' end of one DNA strand.

6.2. Binding PCR products to a microtiter plate

PCR reaction products were mixed with an equal volume of Buffer I (100mM Hepes, 50mM NaCl, 10mM EDTA, pH 7.8) and transferred to individual wells of a streptavidin coated thin wall microtiter plate (Boehringer Mannheim). This was left at room temperature for 1 hour.

6.3. Elution of unbound strand

All liquid volume (containing non-bound DNAs and other reagents) was thoroughly aspirated from the microtiter plate. Without delay, the wells were refilled with 50ml 0.1M NaOH and left at room temperature for 5 minutes. The NaOH solution including the non-biotinylated DNA strand (now denatured from the bound strand) was then thoroughly removed.

6.4. Neutralization of reaction sample

Without delay, the wells were refilled with 50ml Buffer I including SYBR Green I dye (1:10,000 fold dilution).

6.5. Hybridization of the first allele specific oligonucleotide probe

30pmol of 'T Probe' (presented figure 1 as 'DNA Sequence 4') was added to each well in a volume of 1ml water. Optical caps (Perkin Elmer) were used to seal each well, and the plate and its contents were heated to 60 ^{o}C and cooled steadily over ~15 minutes to room temperature. This was achieved upon a TouchDown™ Temperature Cycling Device (Hybaid Ltd).

6.6. Removal of unbound probe

The optical caps were removed, and all liquid volume was thoroughly aspirated from the microtiter plate. The wells were then refilled with 50µl of Buffer I including SYBR Green I dye (1:10,000 fold dilution), and the optical caps were replaced.

6.7. Signal detection procedure

The microtitre plate was placed into a Perkin Elmer 7700 (Taqman) device, and a heating phase applied involving traversing from 35 ^{o}C to 80 ^{o}C at a steady rate of 0.1 ^{o}C per second. During this heating phase, the Taqman device repeatedly excited the samples with an argon laser light source (filtered at 488nm) and collected the

fluorescence that was emmitted at a frequency range of 545-550nm. Data points were collected at 7 second intervals for every well.

6.8. Reprobing with a second allele specific probe

Steps 3-8 above were repeated, this time replacing the 'T Probe' used in step 5 with the 'C Probe' (presented figure 1 as 'DNA Sequence 5').

6.9. Data analysis and interpretation of results

Primary data was plotted on a fluorescence data versus time graph for all wells and for both probe interrogations. This data was 'smoothed' by plotting average fluorescence values determined from a sliding window of 8 data points. The resulting 'primary' data is shown in figures 2a and 2b . A negative differential (derivative) curve of this graph was then plotted and this is shown in figures 3a and 3b. A differential curve of the negative first differential curve was then plotted to give the second derivative shown in figures 4a and 4b.

In figures 3a and 3b, high (H) and low (L) temperature peaks can be seen indicating points of maximal rate of denaturation. These represent DASH signals for matched and one-base mismatched probe-target DNA duplexes respectively. In the second differential (figure 4) these points can be inferred from the points at which the curves cross the X axis. They are also visible in the primary data (figure 2), but can be hard to discern in this representation of DASH results.

Samples X and Y are seen to have only one matched (high temperature) peak in one negative first differential graph and only one mismatched (low temperature) peak in the other negative first differential graph. This indicates that they are homozygous samples. The probing during which X and Y gave a high temperature (matched) peak indicates which allele they contain. Thus, since X gave a high temperature peak with the 'T

Probe', it is an 'A' allele homozygote. Conversely, Y gave a high temperature peak with the 'C Probe', and so is a 'G' allele homozygote.

Sample Z behaved differently to samples X and Y. It gave both high and low temperature peaks with the 'C Probe', and high and low temperature peaks (merged due to proximity into a single wide peak) with the 'T Probe'. Thus, this DNA sample must have both probe allele complementary sequences present within it. Hence it can be deduced to be a heterozygous sample containing both the 'A' and the 'G' alleles.

Figure 1. DNA sequences for use in a DASH assay for scoring alleles of a human NDUFB4 gene polymorphism

DNA Sequence 1 (Sequence listing SE1)

5':

CTGCATTTTGGCACAACCCACC(G/A)TACAACTGACAAACAGGAATGAAAC

:3'

This is a 48 base pair genomic DNA sequence representing a portion of the human NDUFB4 gene. A bi-allelic single nucleotide polymorphism (G to A) is shown in parentheses towards the centre of the sequence.

DNA Sequence 2 (Sequence listing SE2)

5': (Biotin)-CTGCATTTTGGCACAACCC :3'

This is a 19 base oligonucleotide sequence designed for use as 'PCR Primer 1' in a DASH assay for detection of alleles of the polymorphism shown in DNA sequence 1. It carries a biotin moiety attached to the 5' end.

DNA Sequence 3 (Sequence listing SE3)

5': GTTTCATTCCTGTTTGTCAGT :3'

This is a 21 base oligonucleotide sequence designed for use as 'PCR Primer 2' in a DASH assay for detection of alleles of the polymorphism shown in DNA sequence 1.

DNA Sequence 4 (Sequence listing SE4)

5': AGTTGTACGGTGGGT :3'

This is a 15 base oligonucleotide sequence designed for use as the 'C Probe' in a DASH assay for detection of the 'G' allele of the polymorphism shown in DNA sequence 1.

DNA Sequence 5 (Sequence listing SE5)

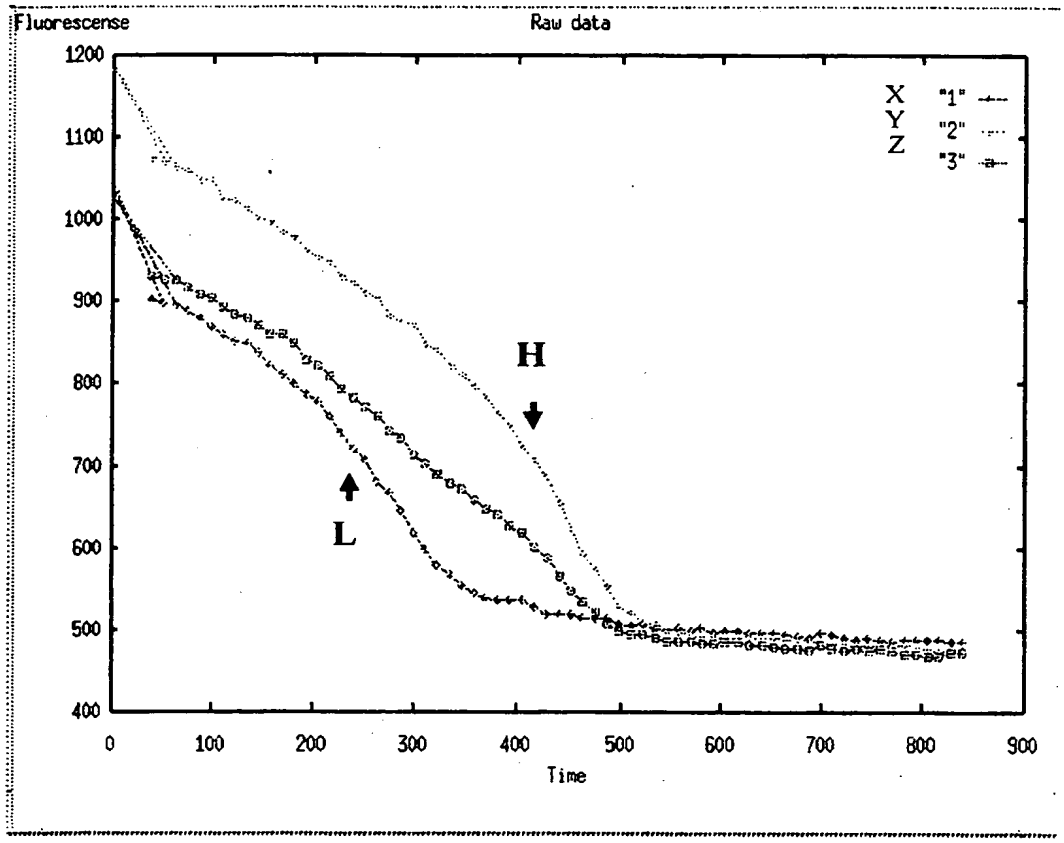
5': AGTTGTATGGTGGGT :3'

This is a 15 base oligonucleotide sequence designed for use as the 'T Probe' in a DASH assay for detection of the 'A' allele of the polymorphism shown in DNA sequence 1.

Figure 2. Primary DASH assay data for an NDUFB4 gene polymorphism

('H' and 'L' indicate points of maximum denaturation rates for matched and mismatched probe-target duplexes respectively. X, Y, and Z are the sample DNAs).

a. Results for the 'C Probe'



b. Results for the 'T Probe'

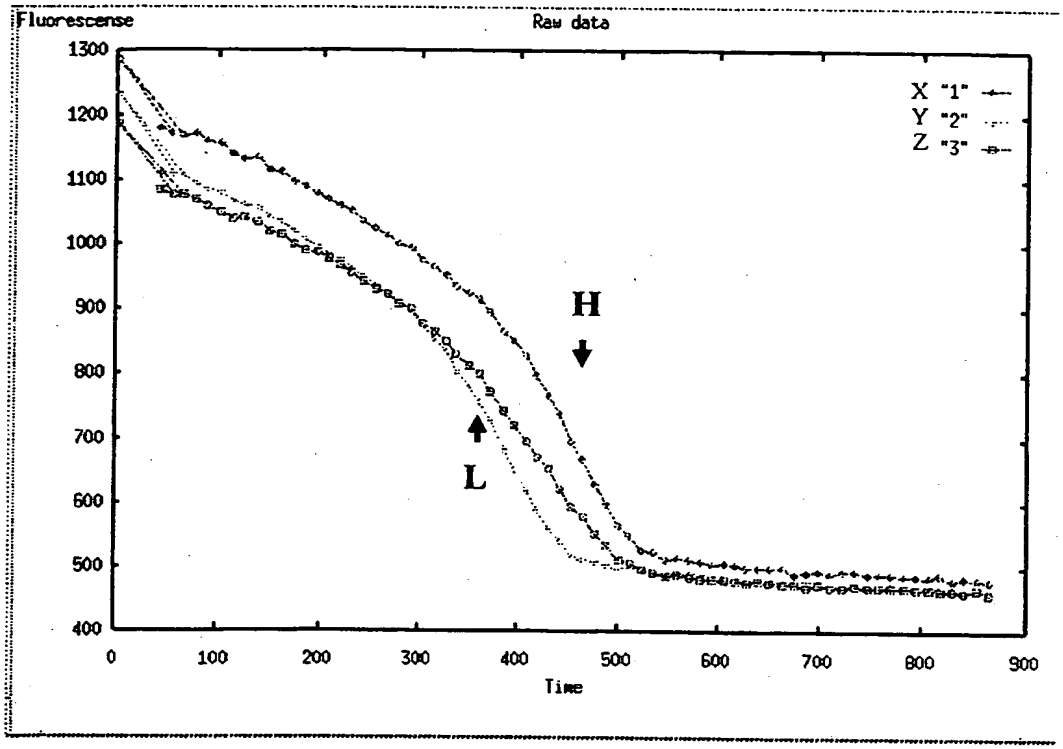
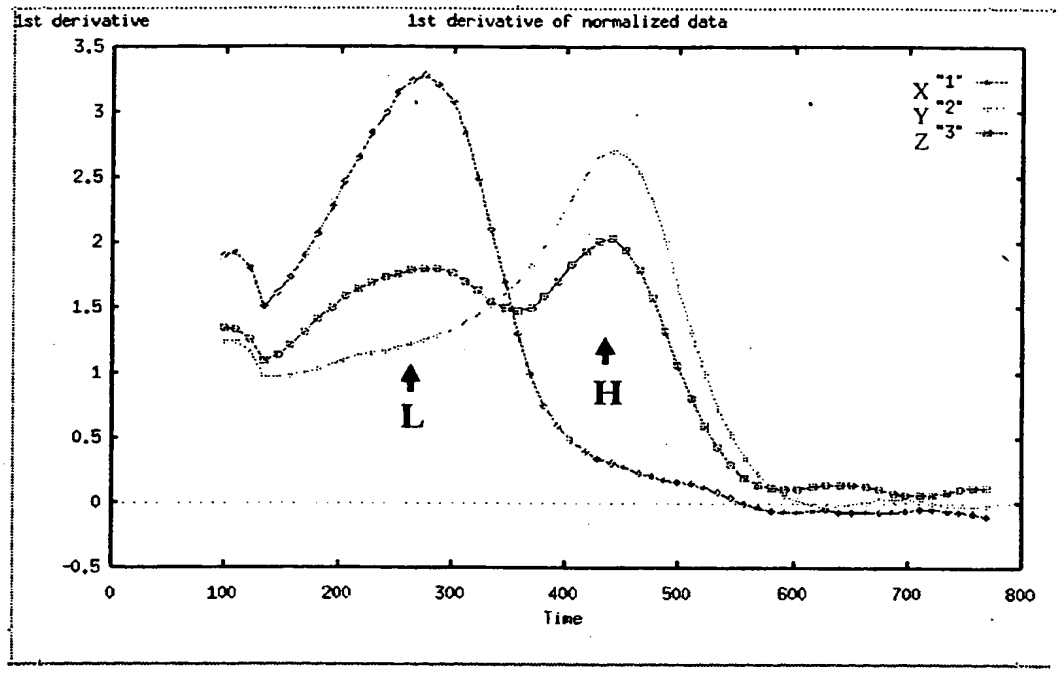


Figure 3. Negative 1st derivative DASH assay data for an NDUFB4 gene polymorphism

('H' and 'L' indicate points of maximum denaturation rates for matched and mismatched probe-target duplexes respectively. X, Y, and Z are the sample DNAs).

a. Results for the 'C Probe'



b. Results for the 'T Probe'

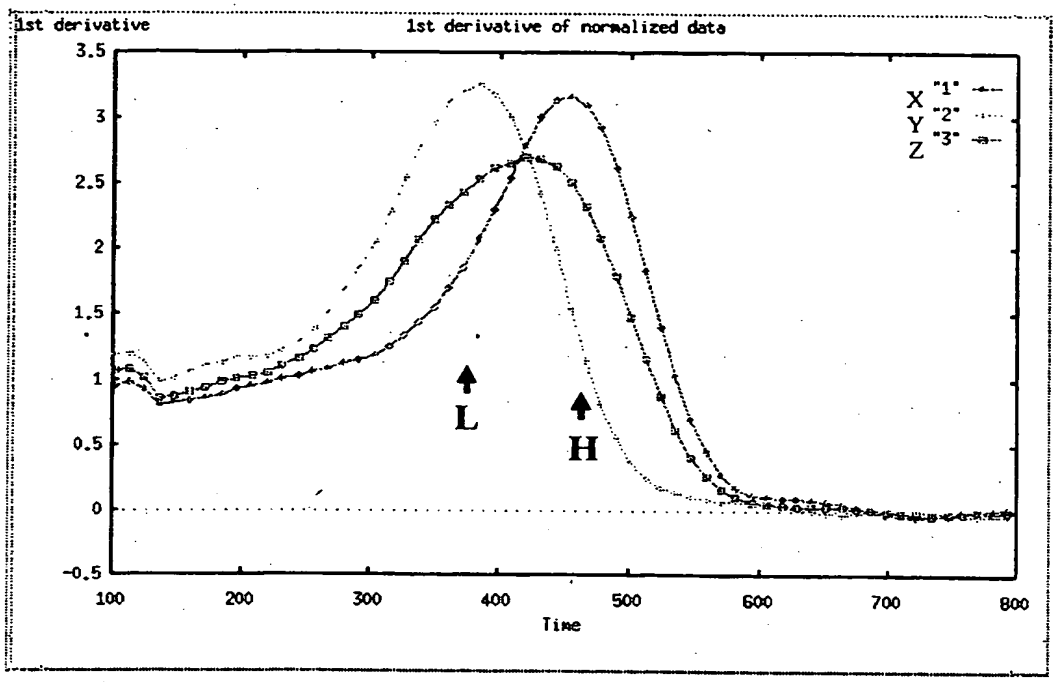
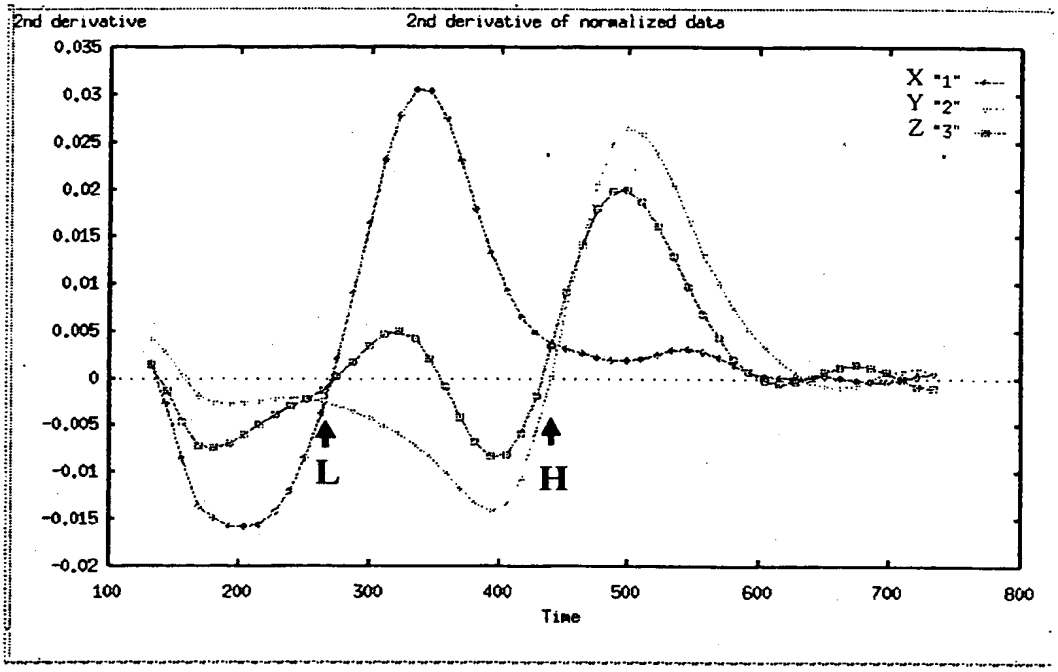


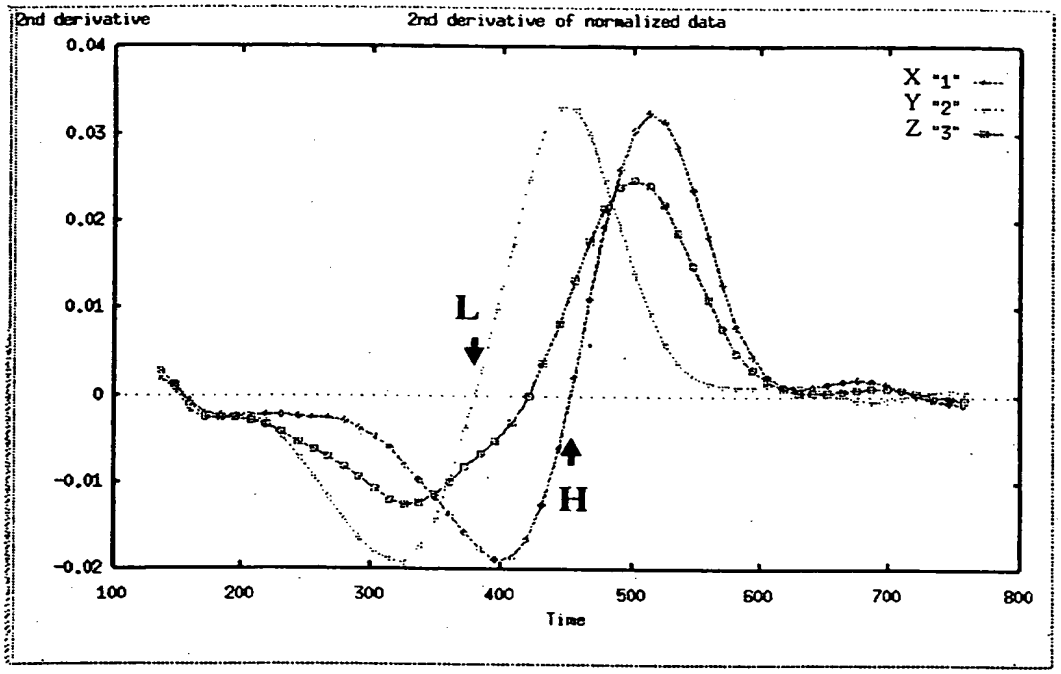
Figure 4. 2nd derivative DASH assay data for an NDUFB4 gene polymorphism

('H' and 'L' indicate points of maximum denaturation rates for matched and mismatched probe-target duplexes respectively. X, Y, and Z are the sample DNAs).

a. Results for the 'C Probe'



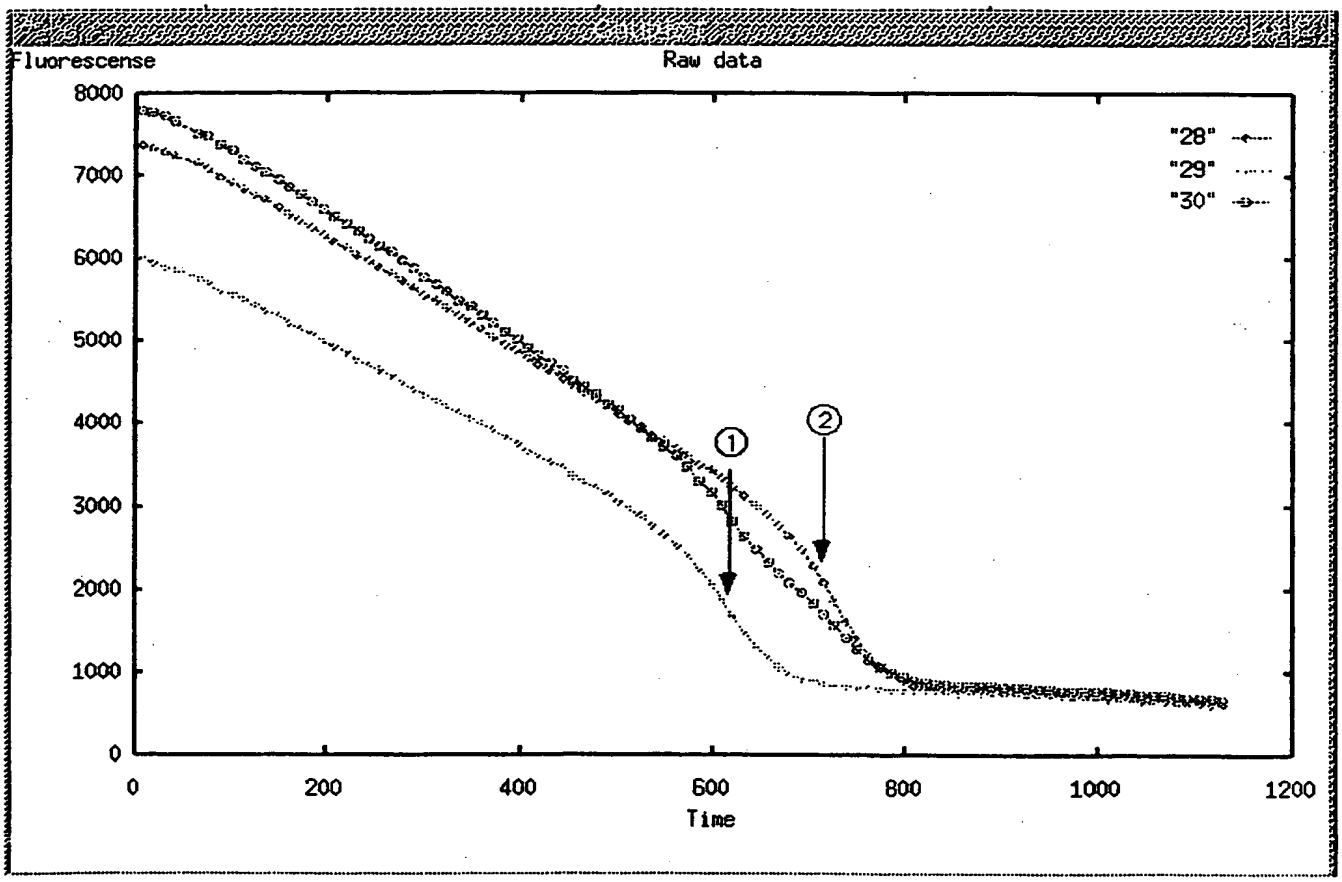
b. Results for the 'T Probe'



7. Appendix

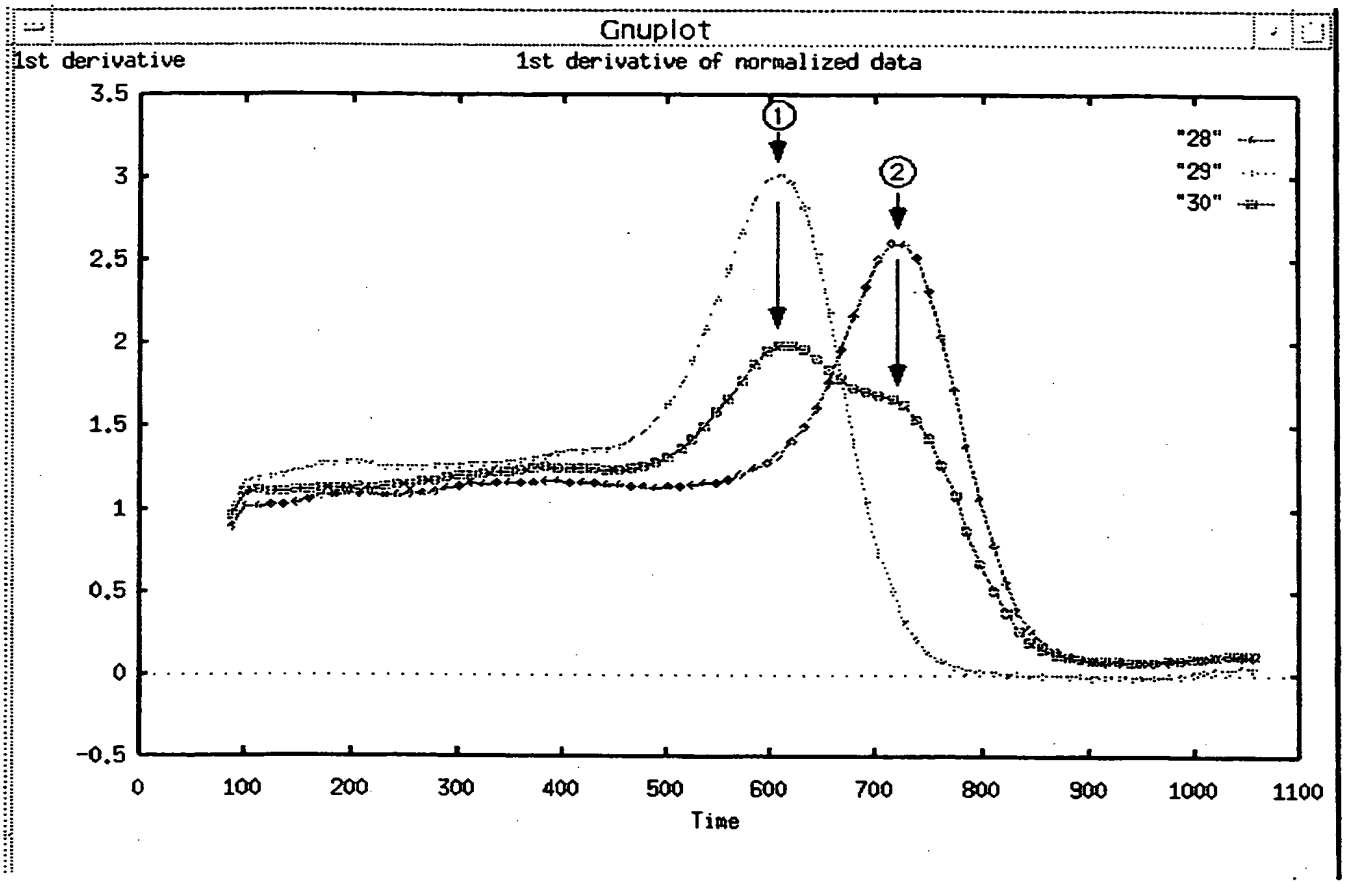
7.1. Example Graphs

7.1.1. Primary data



The above graph shows data for 3 different DNA samples assayed with one allele specific probe. Samples "28, 29, and 30" illustrate typical results for homozygous match, mismatch, and heterozygous samples respectively. Notice that the heterozygous sample 30 exhibits characteristics of both the match and mismatch curves. (1) Tm of mismatched probe/target duplex. (2) Tm of matched probe/target duplex.

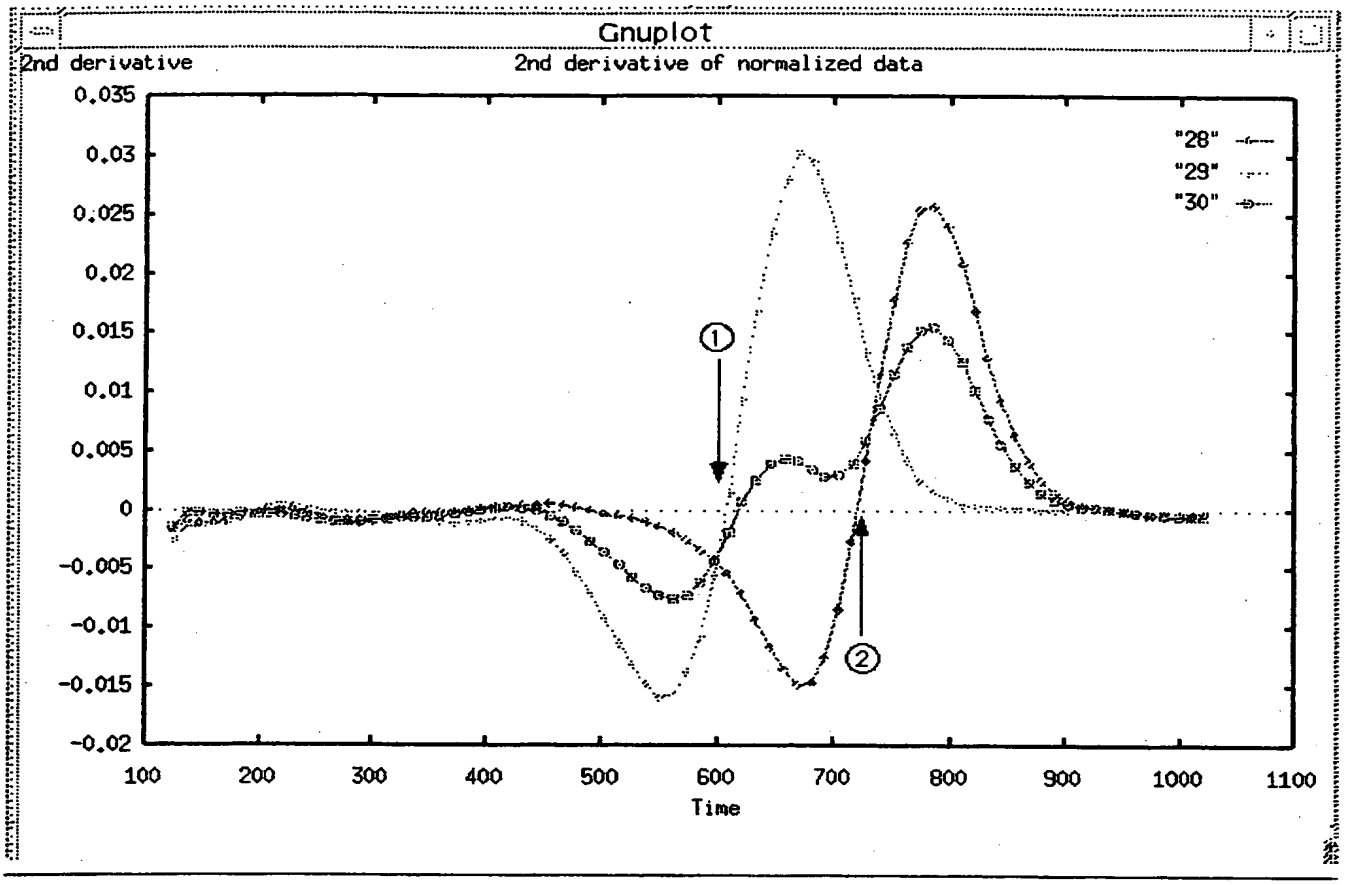
7.1.2. Negative First derivative



5

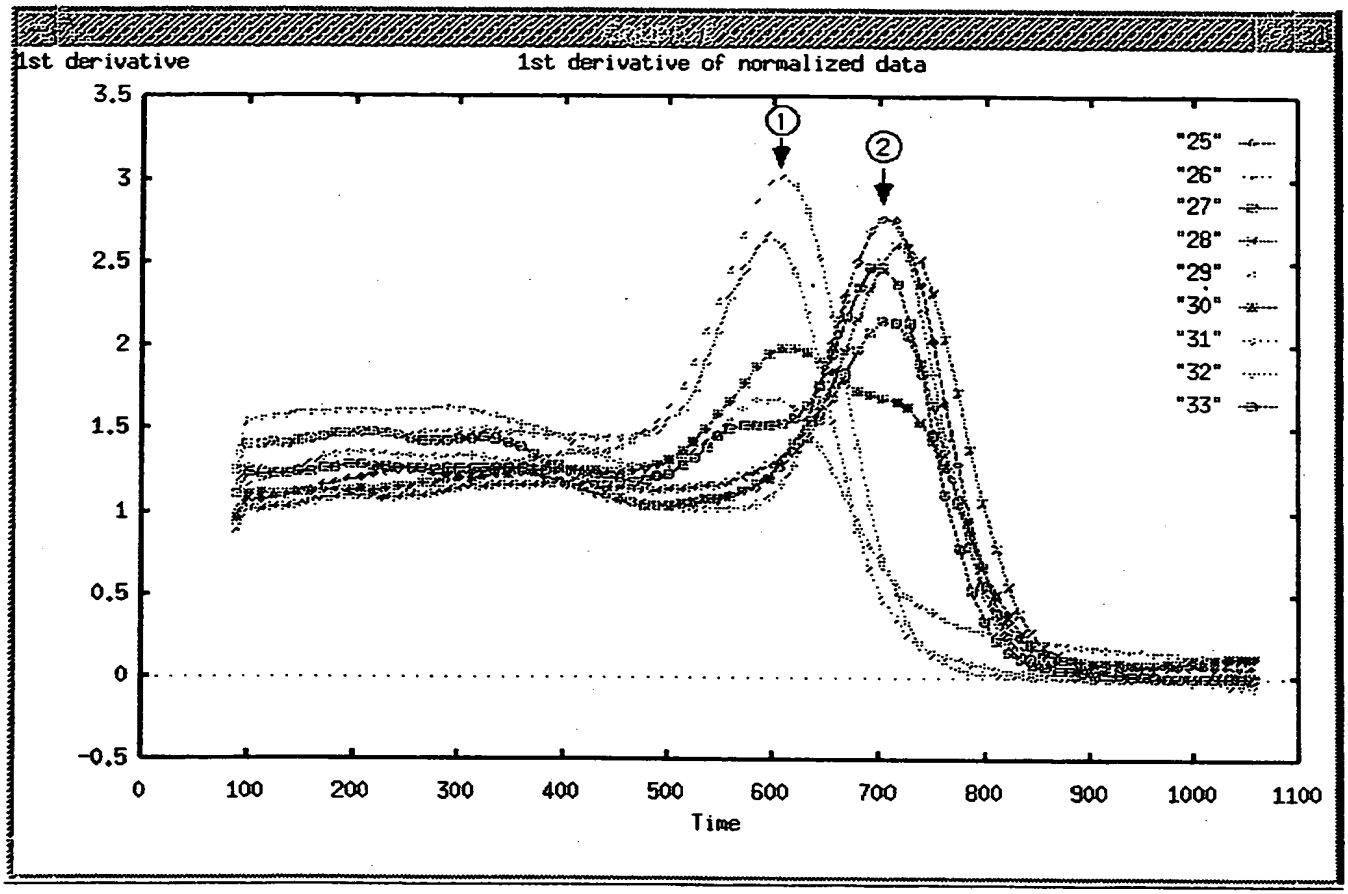
The negative first derivative of three DNA samples are shown. The DNA samples are probed with one allele specific probe. Samples 28, 29, and 30 are homozygous match, homozygous mismatch, and heterozygous, respectively, for the probe allele. (1) Tm of mismatched probe/target duplex. (2) Tm of matched probe/target duplex.

7.1.3. Second derivative



Second derivative data is shown for 3 DNAs. Samples 28, 29, and 30 are homozygous match, homozygous mismatch, and heterozygous. (1) Tm of mismatched probe/target duplex. (2) Tm of matched probe/target duplex.

7.1.4. Normalisation of Tms by Hepes for three different variations



Samples 25-27, 28-30, and 31-33 are negative first derivative sets of data for three different variations. Each set was probed with a locus specific 15mer allele specific oligonucleotide probe corresponding to the respective variation being assayed. The G+C content of these probes varied from 40% to 70%. According to melting temperature theory the Tm's of the probe/target duplexes should vary between these different sequences, but the DASH assay conditions with Hepes buffer normalize the data to fixed Tm values. (1) Tm of mismatched probe/target duplex. (2) Tm of matched probe/target duplex.

CLAIMS

1. A method of detecting DNA variation by monitoring the formation or dissociation of a complex consisting of :-

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex,

(c) a marker specific for the duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex,

which comprises continually measuring an output signal indicative of interaction of the marker with duplex formed from the strand (a) and probe (b) and recording the conditions at which a change in reaction output signal occurs which is attributable to formation or dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a).

2. A method of detecting DNA variation which comprises bringing together

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex,

(c) a marker specific for the duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex,

the components (a), (b) and (c) being brought together under conditions in which :

EITHER (i) the component (a) hybridises to component (b) and the complex is formed with component (c)

OR (ii) the components (a) and (b) do not hybridise and the complex with component (c) is not formed,

thereafter steadily and progressively adjusting the conditions of the environment, respectively,

EITHER (i) to denature the formed duplex and cause dissociation of the complex,
OR (ii) to cause formation of the duplex and resulting complex,
and continually measuring an output signal indicative of the extent of hybridisation of (a) and (b) and resulting complex formation with (c)
and recording the conditions in which a change of output signal occurs which is indicative of, respectively (i) dissociation of the complex or (ii) formation of the complex.

3. A method of detecting DNA variation which comprises forming a complex consisting of:

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation hybridised to the single strand (a) to form a duplex, and

(c) a marker specific for the duplex structure of (a) plus (b) and which reacts uniquely when interacting within the duplex, and

continually measuring an output signal of the extent of the resulting reaction of the marker and the duplex whilst steadily increasing the denaturing environment containing the complex, and recording the conditions at which a change in reaction output signal occurs (herein termed the denaturing point) which is attributable to dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a).

4. A method of detecting DNA variation which comprises bringing together

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex,

(c) a marker specific for the duplex structure of (a) plus (b) and which reacts uniquely when interacting within the duplex,

the components (a), (b) and (c) being brought together prior to formation of the defined complex and under conditions in which (a) and (b) do not hybridise, and steadily adjusting the conditions of their environment to cause formation of the duplex and resulting complex, and

measuring an output signal indicative of the occurrence of hybridisation of (a) and (b) (herein termed the annealing point).

5. A method according to any of claims 1 to 4, which comprises forming a series of two or more complexes of the kind defined, each with a probe specific for a different allele of the variation, and observing their respective denaturing or annealing conditions (e.g. denaturing or annealing temperatures) so as to distinguish alleles of the variation plus the homozygous or heterozygous state if appropriate.

6. A method as defined in any of claims 1 to 5, in which the marker is one which fluoresces when intercalated in double stranded DNA.

7. A method according to claim 6, in which the denaturing or annealing point is determined by reference to the first derivative of the fluorescence measurement curve.

8. A method according to claim 6, in which denaturing or annealing point is determined by reference to the second derivative of the fluorescence measurement curve.

9. A method according to any of the preceding claims, in which the single strand is attached to a support material.

10. A method according to claim 9, in which attachment is by a biotin/streptavidin type interaction.

11. A method according to any of the preceding claims, in which the complex is formed by adding the probe and marker to the single strand in an appropriate buffer solution.

12. A method according to claim 10, in which the buffer solution is Hepes buffer.

13. A method according to any of the preceding claims, using a fluorescent intercalating dye, in which the dye is SYBR Green I.

14. A method according to any of the preceding claims, in which the single strand is derived from a double stranded DNA product of PCR amplification of a target sequence.

15. A method according to claim 14, in which the PCR product is at least 100 base pairs in length.

16. A method according to claim 14, in which the PCR product is from 40 to 100 base pairs in length.

SEQUENCE LISTING

<110> HYBAID Ltd
BROOKES, ANTHONY J

<120> DETECTING DNA VARIATION

<130> N8835

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<151> 1998-10-08

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INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 99/03329

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 36096 A (JENSEN MARK ANTON ;TSENG SUSAN YEN TEE (US); DU PONT (US); BASS JA) 20 August 1998 (1998-08-20) see whole doc. esp. claims and examples	1-16
X	EP 0 478 319 A (TOKYO SHIBAURA ELECTRIC CO) 1 April 1992 (1992-04-01) see whole doc. esp. claims	1-16
Y	YGUERABIDE J ET AL: "Quantitative fluorescence method for continuous measurement of DNA hybridization kinetics using a fluorescent intercalator" ANALYTICAL BIOCHEMISTRY, vol. 228, - 1 July 1995 (1995-07-01) pages 208-220, XP002107647 the whole document	1-16

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 2000	06/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax (+31–70) 340–3016	Müller, F

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INTERNATIONAL SEARCH REPORT

Int. Sional Application No
PCT/GB 99/03329

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	WO 93 10266 A (US GOVERNMENT) 27 May 1993 (1993-05-27) the whole document	
A	STIMPSON D I ET AL: "REAL-TIME DETECTION OF DNA HYBRIDIZATION AND MELTING ON OLIGONUCLEOTIDE ARRAYS BY USING OPTICAL WAVE GUIDES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 92, July 1995 (1995-07), pages 6379-6383, XP002912130 ISSN: 0027-8424	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/GB 99/03329

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